

BARBER *et al.*  
Application No.: 10/067,956  
Page 15

PATENT

A29  
transduce the ribozymes into THP-1 cells. Transduced cells were selected using G418, as in Example 1. ICE mRNA levels were assessed by Northern blot analysis, using RT-PCR generated probes (sense 5'-GACCCGAGCTTTGATTGACTCCGT-3' (SEQ ID NO:34), antisense 5'-GGTGGGCATCTGCGCTCTAGGA-3' (SEQ ID NO:35)). The Northern blot and phosphorimage analysis of this experiment was quantified as shown in Table 17. Multiple ribozymes significantly reduced ICE mRNA levels. The greatest reduction was seen with ribozyme ICE13, which produced a 94% reduction in ICE mRNA levels.--

---

Please insert the accompanying paper copy of the Sequence Listing, page numbers 1 to 16, at the end of the application.

#### REMARKS

These amendments insert the required assigned identifiers for sequences disclosed in the Specification and correct errors of a typographical nature. Sequences appearing in the Figures which require inclusion in the Sequence Listing have had assigned identifiers inserted in the appropriate locations in the "Brief Description of the Drawings".

The amendments to Tables 9 and 10 on page 100, in addition, have relocated the names of the restriction endonucleases above the nucleotide sequences into alignment with their respective recognition sites, as indicated by vertical lines.

Applicants request entry of this amendment in adherence with 37 C.F.R. §§1.821 to 1.825. This amendment is accompanied by a floppy disk containing the above named sequences, SEQ ID NOS:1-56, in computer readable form, and a paper copy of the sequence information which has been printed from the floppy disk.

The information contained in the computer readable disk was prepared through the use of the software program "PatentIn" and is identical to that of the paper copy. This amendment contains no new matter.


BARBER *et al.*  
Application No.: 10/067,956  
Page 16

PATENT

Attached hereto is a marked-up version of the changes made to the Specification by the current Amendment. The attached pages are captioned "**VERSION WITH MARKINGS TO SHOW CHANGES MADE.**"

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,

  
Kenneth A. Weber  
Reg. No. 31,677

TOWNSEND and TOWNSEND and CREW LLP  
Two Embarcadero Center, 8<sup>th</sup> Floor  
San Francisco, California 94111-3834  
Tel: (415) 576-0200  
Fax: (415) 576-0300  
KAW:dmw

**VERSION WITH MARKINGS TO SHOW CHANGES MADE**

Paragraph beginning at line 4 of page 8 has been amended as follows:

A ribozyme "recognition sequence" is the portion of a nucleic acid encoding the ribozyme which is complementary to a target RNA. Upon binding of the ribozyme to the target RNA via this recognition sequence, two regions of double-stranded RNA are formed, termed "helix 1" and "helix 2." A GUC ribozyme typically cleaves an RNA having the sequence 5'-NNNNN\*GUCNNNNNNNNN (~~SEQ ID NO:1~~) (where N\*G is the cleavage site and where N is any of G, U, C, or A) where helix 1 is defined as the 6 to 10 bases 3' of the GUC and helix 2 is defined as the 4 bases 5' of the GUC. GUA ribozymes typically cleave an RNA target sequence consisting of NNNNN\*GUANNNNNNNNN (~~SEQ ID NO:2~~) (where N\*G is the cleavage site and where N is any of G, U, C, or A). A "GUA site" is an RNA sub-sequence that includes the nucleic acids GUA which is cleaved by a GUA ribozyme. A "GUC site" is an RNA sub-sequence which includes the nucleic acids GUC which is cleaved by a GUC ribozyme. A library of GUC hairpin ribozyme-encoding genes will therefore have the subsequence 5'-(N)<sub>(6-10)</sub>AGAA(N)<sub>4</sub>3' (SEQ ID NO:42), where N can be either G, T, C, or A.

Paragraph beginning at line 10 of page 14 has been amended as follows:

The term "tetraloop" refers to a stabilizing modification of loop 3 of the hairpin ribozyme. The standard GUU loop 3 of the hairpin ribozyme (Hampel *et al.* (1990) *Nucl. Acids Res.* 18: 299-304) is replaced by a 12 nucleotide tetraloop sequence, 5'-GGAC(UUCG)GUCC-3' (SEQ ID NO:1) (~~SEQ ID NO:—~~), commonly found in cellular RNA structures. The resulting tetraloop ribozyme has a 7 bp helix 4 (versus 3 in the conventional hairpin ribozyme) and a new UUCG sequence in loop 3. The tetraloop

forms a very stable structure which simultaneously enhances the stability of the ribozyme and decreases the size of loop 3, which is otherwise exposed to cellular nucleases.

Paragraph beginning at line 19 of page 14 has been amended as follows:

Figure 1 illustrates the hairpin ribozyme (SEQ ID NO:45). The hairpin ribozyme consists of a 50 to 54 nucleotide RNA molecule (shaded, in uppercase letters) which binds and cleaves an RNA substrate (lowercase letters). The catalytic RNA folds into a 2-dimensional structure that resembles a hairpin, consisting of two helical domains (Helix 3 and 4) and 3 loops (Loop 2, 3 and 4). Two additional helices, Helix 1 and 2, form between the ribozyme and its substrate. Recognition of the substrate by the ribozyme is via Watson-Crick base pairing (where N or n = any nucleotide). The length of Helix 2 is fixed at 4 basepairs and the length of Helix 1 typically varies from 6 to 10 basepairs. The substrate contains a GUC in Loop 5 for maximal activity, and cleavage occurs immediately 5' of the G as indicated by an arrow. The catalytic, but not substrate binding, activity of the ribozyme can be disabled by mutating the AAA in Loop 2 to CGU.

Paragraph beginning at line 30 of page 14 has been amended as follows:

Figure 2 shows a schematic of trans cleavage and ligation (SEQ ID NOS:46-49). The auto-catalytic ribozyme library is transcribed *in vitro* and allowed to self-cleave. Self-cleaved, helix 2-charged ribozymes are purified and incubated with the target RNA. Following cleavage of target, a portion of the charged ribozymes will ligate themselves to the cleavage products. These product-ribozyme species are then amplified by reverse transcription and PCR to yield the target specific ribozymes.

Paragraph beginning at line 12 of page 15 has been amended as follows:

Figure 5 illustrates the PCR cloning scheme for production of a high complexity ribozyme gene library (P3 and P2 ribozyme sequences = SEQ ID NOS:50-52).

Paragraph beginning at line 23 of page 15 has been amended as follows:

Figure 12 illustrates the Scheme for the construction of ERL030398 (NNNNNNNAGAAVNNN = SEQ ID NO:47).

Paragraph beginning at line 26 of page 16 has been amended as follows:

Figure 28 illustrates several 5' and 3' auxiliary sequences (SEQ ID NO:53-56) that can be used to enhance ribozyme activity.

Paragraph beginning at line 17 of page 62 has been amended as follows:

If the target RNA has a 5' methyl-G cap (such as cellular mRNA and many viral RNAs), the RNA can be immunoprecipitated using monoclonal antibodies directed against the cap structure (Garcin and Kolakofsky (1990); Weber, 1996) and immobilized on Protein G sepharose beads (Pharmacia, Uppsala, Sweden) (see Figure 3). If the target RNA is not capped (such as some viral RNAs, non-messenger cellular RNA or RNA transcribed *in vitro*), it can be bound to streptavidin-agarose beads (Pierce, Rockford Il) via a 30-mer oligonucleotide that is biotinylated at its 3' end (see Figure 3). The sequence of the 30-mer is complementary to the 5' end of the target RNA. If the target is a known viral or cellular RNA, the oligo is designed based on the known sequence of the RNA's 5' end. If the target RNA comes from genomic DNA of unknown sequence that has been converted to RNA via retrovirus packaging, the oligo is

designed based on the retroviral-specific immediate 5' sequence transcribed from the LTR. Likewise DNA cloned into *in vitro* transcription vectors and transcribed by T7 RNA polymerase to yield the target, are engineered to contain specific 30 nt at their 5' end, upstream of the actual target sequence. In general, then, the 3' end of the specific 30-mer biotinylated oligo is bound to the streptavidin column and the 5' 30 nt bind the target RNA by Watson-Crick base pairing (see Figure 3). To prepare the column, the biotinylated oligo is incubated with the beads and unbound oligo is washed out. The target RNA is then mixed with the oligo column, heated to 95° C and cooled slowly to allow annealing of the oligo and target RNA. The column is then washed to remove unbound target RNA.-

Paragraph beginning at line 23 of page 65 has been amended as follows:

If multiple rounds of selection on the same column still yield false positives due to release of inactive ribozymes bound downstream of an active one, the selected ribozymes are then applied to another column prepared with the RNA target bound to the column in the reverse orientation (*i.e.* if target bound on 5' ~~5~~— previously, then switch to 3' ~~3~~— immobilization). This re-screening and amplification is repeated as many times as necessary to satisfy pre-determined requirements set for the ribozymes to be selected (*i.e.* diversity of ribozyme number, ribozyme efficiency, total ribozyme number, etc.) If <sup>32</sup>P ~~P-32~~ UTP is included in the ribozyme transcripts, as mentioned previously, the binding ratio of those ribozymes which remain bound to the target RNA on the column relative to that which has cleaved the target RNA can be tracked from screening to screening. Again, as selection progresses, this ratio will steadily shift greater for ribozymes which cleave the target RNA instead of remaining bound to the target. Furthermore, screening success can be quantified by the number of PCR cycles required to amplify the selected ribozymes (Conrad *et al.* (1995) *Molecular Diversity* 1:69). As the ribozyme pool is further selected and amplified, the number of required PCR cycles would be expected to reduce proportionally.

BARBER *et al.*  
 Application No.: 10/067,956  
 Page 21

PATENT

Paragraph beginning at line 10 of page 71 has been amended as follows:

A fragment comprising an AAV 3' ITR, a tRNAval promoter, and ribozyme library genes was produced by PCR using the primers set P1 and P2 where P1 is a 3' AAV-ITR primer (41 nt) (5'- AGG AAG ATC TTC CAT TCG CCA TTC AGG CTG CGC AAC TGT TG-3' (SEQ ID NO:2) (~~SEQ ID NO: 1~~) and P2 is a 5'- oligonucleotide with sequences for a tRNAval promoter and ribozyme library genes (72 nt) (5'-ATA CCA CAA CGT GTG TTT CTC TGG TNN NNT TCT NNN NNN NGG ATC CTG TTT CCG CCC GGT TTC GAA CCG GGG-3') (SEQ ID NO:3).

Paragraph beginning at line 17 of page 71 has been amended as follows:

A fragment comprising an AAV 5' ITR, a ribozyme library gene, and a neo selection marker was produced by PCR using the primers set P3, an oligonucleotide containing ribozyme library gene complementary to the P2 oligonucleotide (72 nt) 5'- CCC CGG TTC GAA ACC GGG CGG AAA CAG GAT CCN NNN NNN AGA ANN NNA CCA GAG AAA CAC ACG TTG TGG TAT-3' (SEQ ID NO:4) (~~SEQ ID NO: 1~~) and P4 a 5' AAV-ITR primer (40 nt) (5'-AGG AGA TCT GCG GAA GAG CGC CCA ATA CGC AAA CCG CCT C-3' (SEQ ID NO:5) (~~SEQ ID NO: 1~~).

Paragraph beginning at line 18 of page 73 has been amended as follows:

More specifically, p1014-2k (100 :g) was thoroughly digested overnight at 37°C with restriction enzymes BamHI and MluI (200 units each). The digested DNA was fractionated by agarose gel electrophoresis. An 8 kb fragment was extracted from the gel. 0.2 pmol of the 8 kb fragment was ligated with 3 oligonucleotides: (~~oligo 1:~~ Oligo 1: 5'-pGAT CCA CCC CCC NNN NNN NAG AAN NNN ACC AGA GAA ACA CAC GTT GTG GTA TAT TAC CTG GTA-3' (SEQ ID NO:6) (~~SEQ ID NO: 1~~), Oligo 2: ~~Oligo2:~~ 5'-pGGG GGG TG-3' (~~SEQ ID NO: 1~~), and Oligo 3: 5'-pCGG GTA CCA

BARBER *et al.*  
 Application No.: 10/067,956  
 Page 22

PATENT

GGT AAT ATA C-3' (SEQ ID NO:7) (~~SEQ ID NO: —~~) as illustrated in Figure 8 at a molar ratio of 1:3:30:30 (8kb fragment: oligo1: oligo2: oligo3). Ligation was performed using 10 units of ligase at 16°C overnight. All of the oligonucleotides were phosphorylated at the 5' end to ensure high ligation efficiency.

Paragraph beginning at line 25 of page 74 has been amended as follows:

To assure that the hygromycin resistant gene copied by PCR has the right sequence, plasmid pAAV/hygro was transfected into HeLa cells followed by hygromycin selection. Once the resistance to hygromycin was confirmed, a DNA fragment containing the U5 ribozyme transcription unit under the control of PGK promoter was cut from plasmid pPolII/PGKmus/neoBHGPA (Figure10) and cloned into pAAV/hygro such that the transcription of the hygromycin resistance gene and that of ribozyme are towards opposite directions. Afterward, a 3 kb DNA fragment was used to replace the BamHI and MluI fragment of U5 ribozyme-coding region. The resulting plasmid pAAVhygro-PGK was digested completely with BamHI and MluI and gel purified. Three oligonucleotides: Oligo 4: 5'-pAAT TCT GCA GAT ATC CAT CAC ACT GGC GGG GAT CCT CGA GNN NNN NNN AGA ANN NNA CCA GAG AAA CAC ACG GAC TTC GGT CCG TGG TAT ATT ACC TGG TA-3' (SEQ ID NO:8) (~~SEQ ID NO: —~~), Oligo 5: 5'-pCTC GAG GAT CCC CGC CAG TGT GAT GGA TAT CTG CAG-3' (SEQ ID NO:9) (~~SEQ ID NO: —~~), and Oligo 6: 5'-pGCG TAC CAG GTA ATA TAC CAC GGA CCG AAG TCC GTG TGT TTC TCT GGT-3' (SEQ ID NO:10) (~~SEQ ID NO: —~~) were then ligated to the linearized vector according to the protocol described above to generate pAAVhygro-pGK-lib. The complexity of the ribozyme library containing 8 randomized nucleotides in helix 1 and 4 nucleotides in helix 2 is  $4^{4+8}$ ,  $2 \times 10^7$ . The number of individual bacterial colonies in the library is  $8 \times 10^7$ , which is the about 98% of chance of having  $2 \times 10^7$ .



BARBER *et al.*  
 Application No.: 10/067,956  
 Page 23

PATENT

Paragraph beginning at line 24 of page 75 has been amended as follows:

The expression of neo<sup>r</sup> in Hela cells was tested for plasmid p1016 to assure that the neo<sup>r</sup> was not mutated. After digestion with BamHI and MluI, the 8 Kb fragment containing p1016 backbone was ligated with 3 oligonucleotides: Oligo 7: 5'-pCGA AAC CGG GCG GAA ACA GGA TCC NNN NNN NNA GAA NNN NAC CAG AGA GAA ACA CAC GGA CTT CGG TCC GTG GTA TAT TAC CTG GTA-3' (SEQ ID NO:11) (~~SEQ ID NO: —~~), Oligo 8: 5'-pGGA TCC TGT TTC CGC CCG GTT T-3' (SEQ ID NO:12) (~~SEQ ID NO: —~~), and oligo 3: 5'-pCGC GTA CCA GGT AAT ATA CCA CGG ACC GAA GTC CGT GTG TTT CTC TGG T-3' (SEQ ID NO:13) (~~SEQ ID NO: —~~) to generate pAAVlib by the method described above.

Paragraph beginning at line 12 of page 77 has been amended as follows:

To construct the EBV plasmid ribozyme library, we obtained plasmid vector pREP4 from Invitrogen, that contains the EBV EBNA-1 gene and the EBV origin of replication as well as a hygromycin resistant gene expression cassette driven by the HSV TK promoter. A ribozyme cassette, U5 ribozyme against HIV1 (Mang et al. (1994) *Proc. Natl. Acad. Sci. USA*, 90: 6340-6344) driven by tRNA promoter, was placed in the polylinker region of pREP4. The resulting plasmid was named pEBVU5. Plasmid pEBVU5 contains an unique Bam HI site right in front of the helix I of ribozyme and unique Eco RV site about 735 basepairs down stream of the ribozyme sequence. The ribozyme library was generated by PCR reaction using the pEBVU5 as template with two primers, libbam and EBVlibeco (Figure 12). The primer libbam contains degenerated oligonucleotide in the helix I and helix II of ribozyme sequence. The sequences of these two primers are libbam (5'-CCC CCG GGG GAT CCN NNN NNN NAG AAV NNN ACC AGA GAA ACA CAC GGA CTT CGG TCC GTG GTA TAT TAC CTG GTA CGC GTT TTT GCA TTT TT-3' (SEQ ID NO:14) (~~SEQ ID NO: —~~)) and EBVlibeco

BARBER *et al.*  
 Application No.: 10/067,956  
 Page 24

PATENT

(5'-TGG GGT GGG AGA TAT CGC TGT TCC TTA-3' (SEQ ID NO:15)(~~SEQ ID NO:~~ ⇒)).

Paragraph beginning at line 30 of page 79 has been amended as follows:

To create the ribozyme library insert, three oligonucleotides were annealed in annealing buffer (50mM NaCl, 10mM Tris pH 7.5, 5mM MgCl<sub>2</sub>) at a molar ratio of 1:3:3 (oligo1:oligo2:oligo3) by heating to 90°C for 5 minutes followed by slow cooling to room temperature as shown in Figure 14. The oligonucleotides were Oligo1, 5'-pCGC GTA CCA GGT AAT ATA CCA CGG ACC GAA GTC CGT GTG TTT CTC TGG TNN NNT TCT NNN NNN NNG GAT CCT GTT TCC GCC CGG TTT-3' (SEQ ID NO:16)(~~SEQ ID NO:~~), Oligo2, 5'-pGTC CGT GGT ATA TTA CCT GGT A-3' (SEQ ID NO:17)(~~SEQ ID NO:~~), and Oligo3, 5'-pCGA AAC CGG GCG GAA ACA GG-3' (SEQ ID NO:18)(~~SEQ ID NO:~~).

Paragraph beginning at line 31 of page 97 has been amended as follows:

After multiple ribozymes have been identified to be responsible for the selected phenotype, primers will be designed to match the target sequence (sense sequences) of the ribozymes as well as the antisense sequences. For example, if the cloned ribozyme contains a sequence: 5'-AAAAUUUUagaaGCGG-3' (SEQ ID NO:19), 5'-AAAAUUUUagaaGCGG, (~~SEQ ID NO:~~) where the underlined nucleotides indicate the regions of a ribozyme forming helixes with the target RNA, the primer that matches the sense sequence will be 5'-CCGCngtcAAAATTTT-3' (SEQ ID NO:20) ~~5'-CCGCngtcAAAATTTT3' (SEQ ID NO:     )~~ and the one that matches the antisense sequence will be 5'-AAAATTTTGACnGCGG-3' (SEQ ID NO:21) ~~5'-AAAATTTTGACnGCGG-3'.~~

BARBER *et al.*  
 Application No.: 10/067,956  
 Page 25

PATENT

Paragraph beginning at line 19 of page 98 has been amended as follows:

The RSTs consisted of 15 to 16 ribonucleotides with one additional degenerate ribonucleotide at the 4th position from 5' end. Such RSTs sequences are not good primers/probes for DNA PCR or southern hybridization assays that are normally employed for identification of full length cDNA from short DNA sequences. To circumvent the problem, we designed a degenerate primer based from the known RSTs (*e.g.*, RRRR nGTC RRRRRRRNNNN 3', SEQ ID NO:43 ~~SEQ ID NO:~~  ).

Paragraph beginning at line 2 of page 99 has been amended as follows:

Poly A mRNA isolated from parental cells and the selected cells is used as templates. Reverse transcription PCR (RT-PCR) is performed using the polyT primer: 3'-NTTTTTTTTTTTT<sub>(20)</sub>CGAGGGTGAAGTCTAACCATTGT-5' (SEQ ID NO:22) 3' NTTTTTTTTTTTTT<sub>(20)</sub>CGAGGGTGAAGTCTAACCATTGT-5' (SEQ ID NO:     ).

Paragraph beginning at line 7 of page 99 has been amended as follows:

RST primers and primer 3' CGAGGGTGAAGTATAACCATTGT 5' (SEQ ID NO:23) is used to specifically amplify cDNA containing RST sequences.

Paragraph beginning at line 17 of page 99 has been amended as follows:

The isolation of one or more ribozymes from the library, based on their conferred phenotype, gives us a probe that can be used to clone the target gene. The probe sequence, or ribozyme sequence tag (RST), consists of 16 bases, 15 of which are specific for the target RNA. To illustrate the conversion from the sequence of an isolated ribozyme to an RST, an example of a ribozyme against PCNA mRNA is used. A ribozyme known to cleave PCNA mRNA has the sequence 5'--

GAGCCCUGAGAAGGCG--3' (SEQ ID NO:24), where the underlined bases are the arms of the ribozyme that bind to its target mRNA. An RST is the deduced sequence of the target mRNA, based on the complement of the binding arms of the identified ribozyme, including the requisite GUC required by the hairpin ribozyme. Thus, the RST corresponding to this ribozyme would be: 5'-CGCCNGUCCAGGGCUC-3' (SEQ ID NO:25) (~~SEQ ID NO:~~ ), where N=any of the four bases. Interestingly, previous knowledge of the hairpin ribozyme would have dictated that the N position could not be an A (Anderson et al, (1994) *Nucl. Acid. Res*: 22), however we have found that restriction to be incorrect and may be specific only for the native hairpin ribozyme. Therefore, an RST has the following format: 5'-XXXXNGUCXXXXXXXX-3' (~~SEQ ID NO:~~ ), where X is a specific base (A,C,G or T) based on the complementary sequence of the isolated ribozyme and N is any of the four bases, thus resulting in 15 known bases and one N. This is sufficiently unique in the human genome for accurate target gene identification.

Paragraph (and Table 9.) beginning at line 4 of page 100 has been amended as follows:

To clone the target gene, a specific oligonucleotide is synthesized containing the RST sequence (example below is RST for PCNA ribozyme), a few unique restriction sites (e.g. XbaI, XhoI, EcoRI) and a biotin molecule on the 5' end (Table 9 below).

Table 9. Biotinylated RST Primer (SEQ ID NO:36)

	XbaI	XhoI	EcoRI	
5' -- Biotin-GCATG CTCCT CTAGA CTCGA GGAAT TCGAG CCCTG GACNA GGC -- 3'				
				PCNA RST PRIMER (SEQ ID NO:37)

Occasionally, ribozymes are isolated that target low abundance mRNAs in the target cell. If the target mRNA is scarce enough, the single round of PCR amplification is insufficient to reproducibly detect the PCR product. In these instances, a second round of PCR can be included by adding a polyC tail to the 3' end of the first strand cDNA (see right side of Figure 17). This allows PCR amplification using a polyG primer (5'-GAAGA ATTCT CGAGG GGCCG CGGGI IGGGI IGGGI IGN-3' (SEQ ID NO:40), (GGGII)<sub>3</sub> (SEQ ID NO:44) (GGGII)<sub>3</sub> Primer & Tag-Specific Primer (underlined) (SEQ ID NO:41) (SEQ ID NO:42) and the RST primer prior to digestion and adaptor addition. The polyG stretch also contains inosine residues to prevent the non-specific priming observed when only G residues are used.

Paragraph beginning at line 1 of page 117 has been amended as follows:

Ribozyme sequences can be rescued by adenovirus in the presence of Rap and Cap expressing vector and by wild-type of AAV. Without extensive optimization of the rescue conditions, we got low efficiency of rescue by adenovirus and by wild-type AAV as many other research groups did. Thus, we rescued ribozyme sequences by PCR amplification using primers flanking the ribozyme expressing cassette: 5' PA (5' CCGTTGGTTTCCGTAGTGTAGTGG 3' (SEQ ID NO:26)) and 3' PA (5' GCATTCTAGTTGTGGTTTGTCC 3' (SEQ ID NO:27)). The PCR condition is 94°C for 2 min followed by 30 cycles of 94°C for 30'', 56°C for 30'', and 68°C for 45'' then 68°C for 7' using the expanded long enzymes (BMB) according to the procedure recommended by the manufacture. The PCR products were cloned and sequenced. We have obtained 8 ribozyme sequences from colonies after the first round and second round of replating. To confirm inactivation of tumor suppressor gene expression by their cleavage activity, the individual ribozymes as well as their corresponding disable ribozymes and the control vector were introduced back into the parental U138 cells.

Paragraph beginning at line 15 of page 117 has been amended as follows:

Ribozyme G1 isolated from library leads to the growth of colonies in soft agar. After confirming the correlation between ribozymes and the phenotype change of cells, the ribozyme sequences are used to determine the ribozyme sequence tag (RST). For example: RST sequence 5' GCCA ngtc CCGGGTT 3' (SEQ ID NO:28) is derived from ribozyme sequence 5' AACCCGGagaaTGGC 3' (SEQ ID NO:29). Gene sequences can be identified by genebank search or by methods described in Example G using RST sequences. Three of eight RSTs identified from U138 cells were mapped to a single chromosomal band at which loss of homozygosity are frequently associated with cancers of pancreatic (80%), prostate (30-75%), head and neck (67%), colon (60%), ovarian (50-73%), breast (20-80%), renal (64%), and oral SCC (56%). The soft agar clonogenic assay

BARBER *et al.*  
Application No.: 10/067,956  
Page 29

PATENT

can be applied to any partially transformed cell line which does not grow in soft agar under optimized conditions for the identification of tumor suppressors. For cell lines which have background colonies in soft agar, we can enrich the candidate ribozymes from the library by rescue ribozymes from pooled soft agar colonies by PCR, clone the PCR products in AAV vectors by shotgun cloning and transduction of AAV DNA isolated from pooled bacterial clones for multiple cycles of selection and rescue.

Paragraph beginning at line 13 of page 118 has been amended as follows:

Hairpin ribozyme expression cassettes were synthesized by a PCR mutagenesis reaction using a double stranded DNA tetraloop ribozyme gene as a template (...agaaNNNNACCAGAGAAACACACGGACTTCGGTCCGTGGTATATTACCTGG TACGCGT...) (SEQ ID NO:30), and a mutagenic oligonucleotide containing sequences for the 5' end of the gene, including the target recognition sequences in the ribozyme, as a primer (GATATCGGATCCCAACAACACTAGAACGGCACCCAGAGAAACACACG) (SEQ ID NO:31).

Paragraph beginning at line 2 of page 119 has been amended as follows:

Northern blot analysis was performed to determine the relative levels of IL-1 $\beta$  RNA in ribozyme-expressing and control cells. The probe was prepared from RT-PCR fragments derived from THP-1 RNA (the RT-PCR primers used for probe preparation: sense 5'-CAGAAGTACCTGAGCTCGCCAGTGA-3' (SEQ ID NO:32), anti-sense 5'-GCAGGCAGTTGGGCATTGGTGTAGA-3' (SEQ ID NO:33)), and the authenticity of the fragments was confirmed by multiple restriction digests. The probe was labeled by random priming using the DNA Labeling kit (Pharmacia), and free nucleotides were removed by spin column. As quantified in Table 15, numerous anti-IL-1 $\beta$  ribozymes significantly reduced target IL-1 $\beta$  mRNA levels in THP-1 cells. The degree of mRNA reduction ranged from 45% to 99%.

BARBER *et al.*  
Application No.: 10/067,956  
Page 30

PATENT

Paragraph beginning at line 6 of page 120 has been amended as follows:

IL-1 $\beta$  Convertase (ICE) is an intracellular protease that cleaves the precursor of IL-1 $\beta$ , thereby creating the mature extracellular form of the protein. Ribozymes against ICE were cloned into AMFT vector and rAAV vectors were used to transduce the ribozymes into THP-1 cells. Transduced cells were selected using G418, as in Example 1. ICE mRNA levels were assessed by Northern blot analysis, using RT-PCR generated probes (sense 5'-GACCCGAGCTTTGATTGACTCCGT-3' (SEQ ID NO:34), antisense 5'-GGTGGGCATCTGCGCTCTAGGA-3' (SEQ ID NO:35)). The Northern blot and phosphorimage analysis of this experiment was quantified as shown in Table 17. Multiple ribozymes significantly reduced ICE mRNA levels. The greatest reduction was seen with ribozyme ICE13, which produced a 94% reduction in ICE mRNA levels.